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(57) Abstract

Methods are provided for making real-time determinations of the concentration and for monitoring the structural profile of a target solute in a solution. Non-diffusion bound affinity chromatographic techniques are used to generate chromatograms having sharply defined breakthrough curves. Based on the difference between the sensed equilibrium concentration of the impurities which break through the matrix and the concentration of all solutes in the sample, the concentration of the target solute in a solution can be determined. Alternatively, a chromatogram representative of the structural profile of a protein in a sample comprising a mixture of structural variants may be produced using the method of the invention. The assays may be carried out in real-time so that production or purification of a valuable substance such as a pharmaceutical recombinant protein or the like can be monitored meaningfully.

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QUANTITATIVE ANALYSIS AND MONITORING OF PROTEIN STRUCTURE BY SUBTRACTIVE CHROMATOGRAPHY

Background of the Invention

The present invention generally relates to a method for performing quantitative and structural analyses on solutions containing multiple solutes. In particular, the invention relates to methods for determining the presence and concentration and for characterizing the structural profile of an analyte in a solution utilizing subractive frontal breakthrough analysis.

It promotes efficiency in the production of therapeutic substances to monitor each step of the synthesis process to insure that quality and quantity of a desired intermediate or product is within specification. In addition to there being extensive federal regulation designed to insure the integrity of such processes, economics dictate that proper safeguards be taken to maintain precision with regard to the production of therapeutics. Indeed, in any manufacturing stream which produces a drug, pesticide, food additive, dye, or other high value substance in a multistep process, monitoring product concentration rapidly and accurately at various production or purification stages can be a key to maintaining efficient operation.

While analytical methods exist for precisely quantitatively determining a particular solute such as a protein or organic compound in a mixed solution, due

to the time required to carry out these analyses, and to their cost and complexity, they are impractical for routine use. It is not feasible to use these methods in conjunction with continuous monitoring of a manufacturing process because they do not offer "real time" information. By the time data from a particular sample can be processed, the state of the system may have changed dramatically.

Further, qualitative or structural analysis of structural variants of proteins, e.g., recombinantly produced therapeutic proteins, is not presently possible except in those instances where the variation in the amino acid sequence or tertiary structure results in an altered activity profile or the separate species can be resolved on a gel. Determining the presence of structural variants is particularly important in the field of biosynthetic protein production, e.g., in recombinant DNA technology as applied to the manufacture of therapeutic proteins. Recombinantly produced proteins have a higher rate of expression error, and can include a number of "minor" structural variants having, for example, diminished activity, harmful side effects, or undesirable antigenicity. Structural variants also may be produced during post translational protein modification by, for example, variation in glycosylation pattern, disulfide bonding, or protein folding.

In the biotechnology industry, it is often necessary to monitor the concentration of a product in a mixture which may contain hundreds of contaminating species including cell debris, various solutes, nutrient components, DNA, lipids, polysaccharides, protein species having similar physiochemical

properties, and structural variants of a single recombinantly produced protein. While the concentration of the target product in the harvest liquor is usually on the order of 100 mg/l, it is sometimes as low as 1 mg/l. To complicate identification further, due to the fragility of many target solutes, they must be treated with relatively low fluid shear, and preferably with only minimal and short duration contact with potentially denaturing surfaces.

One known method of identifying solutes in a solution is affinity chromatography, which involves passing a feed mixture over a matrix such as a packed bed of selectively sorptive particles to bind one or a subset of the solutes in the mix. Subsequent passage of solutions that modify the chemical environment at the sorbent surface results in elution of sorbed species. Solutes flow through these systems convectively in the interstitial space among the particles and diffusively within the particles. The media used for liquid chromatography typically comprises soft, highly porous particles having a high surface area to volume ratio. As a result, a liquid chromatography process cannot be run at pressures exceeding about 50 psi, and attempts to increase the fluid velocity are counterproductive to separation. High Performance Liquid Chromatography (HPLC) employs as a matrix rigid porous beads made typically of an inorganic material such as silica or a rigid polymer such as styrene divinylbenzene. HPLC allows somewhat faster and higher resolution separations.

Typically, chromatographic procedures employed for purification involve four steps: loading; washing; eluting; and re-equilibrating. The rate limiting step

in each stage is the transport of molecules between the mobile fluid and the static matrix surface. Optimum efficiency is promoted by rapid, preferably instantaneous mass transfer and high fluid turnover. During sorbent loading, fewer moleculares are sorbed as the velocity of the mobile phase in the bed increases. At some mobile phase velocity, some target solute is lost in the effluent as "breakthrough", i.e., passes through the matrix without binding and appears in the effluent. If the breakthrough concentration is limited to, for example, 5% of the inlet concentration, that limit sets the maximum bed velocity which the bed will tolerate. Further increases in bed velocity thereafter are wasteful and can only serve to decrease loading per unit surface area.

Recently the art has developed novel matrix designs for increasing the speed of separation of liquid chromatography. For example, by using as a matrix a non-porous microparticulate material, one can avoid the rate limiting diffusion step and greatly increase the speed of separation. That is, solution will flow convectively through the interstitial spaces between particles forming the matrix, and solutes will interact at the particle surface exposed at the walls of the interstices. In this manner, a separation can be carried out much more quickly than in "diffusion bound" chromatography systems, but at the cost of greatly diminished capacity.

One can increase chromatographic throughput by using a matrix comprising small porous particles having a relatively large pore diameter, so that convective flow can be induced through, as well as around, the particles. This type of chromatography is referred to

as Perfusive Chromatography and is described in copending application serial number 376,885, filed July 6, 1989, the disclosure of which is incorporated herein by reference. Perfusive chromatographic techniques permit high speed, high capacity, high resolution separation. Perfusive matrices may be purchased from PerSeptive Biosystems, Inc. of Cambridge, Mass.

Even the fastest of the above discussed known chromatographic techniques are too slow to afford a method for real-time analysis of solute in a product stream. Due to the complexity of the solutions being monitored, and the multiple steps required, these known analytical techniques cannot provide data quickly enough to allow meaningful adjustments to be made to a production process, nor are they sensitive enough to detect structural variants of a proteinaceous solute.

It is an object of the invention to provide methods which exploit the benefits of high speed chromatographic techniques to allow real time monitoring of solute concentration in a process liquor. Another object is to provide such methods that can rapidly, accurately, and precisely monitor the concentration of a therapeutic substance or other solute at any stage of a production or purification process. A further object is to provide methods for producing a profile of the mixture representative of the nature and relative concentration of structured variants. Another object is to provide such methods that have a self-checking capability.

Summary of the Invention

The present invention provides a method and apparatus for rapid assay and characterization of therapeutic and other substances based on what is described herein as subtractive chromatography. In accordance with the invention, a solution containing multiple solutes is passed through a matrix having binding sites specific for one or more target solutes. As used herein, "target solute" is broadly defined and encompasses any water soluble analyte but typically is a protein such as a recombinantly produced protein. By analyzing the effluent flowing from the column, the presence and concentration or the profile of the structural variants of the target solute can be determined.

In accordance with a first aspect of the invention, a feed solution containing at least one target solute, for example, a biologically active molecule such as a polypeptide, protein, polysaccharide, or the like, in admixture with other solutes, is passed through a matrix comprising binding sites specific to the target solute. As the feed solution passes through the matrix, the target solute will adsorb at the binding sites, thereby virtually eliminating any concentration of the target solute in the effluent. During this process, a limited amount of non-target solute may also non-specifically adsorb to the matrix. The effluent is monitored to determine its solute concentration. While, in a preferred embodiment of the invention, this will entail monitoring the ultra-violet absorption of the effluent, which is proportional to concentration, it should be understood that any number of alternative

methods can be used to the same effect. Any method which produces data related to the solute concentration of the effluent is suitable.

As the effluent begins to flow from the column, the concentration of contaminating or "non-target" solute(s) in the effluent will increase until the concentration of non-target solute in the effluent reaches an equilibrium level equal to the concentration of non-target solute in the feed. When graphed as the relationship between, for example, ultra-violet absorption and time, this stage of the assay procedure will result in an upturned slope or vertical line, depending on the nature of the matrix, which develops into a flat, horizontal line as solute concentration in the effluent maximizes.

The equilibrium concentration of solutes in the effluent will remain substantially constant as the feed solution is passed through the matrix as long as binding sites remain available. Eventually, however, the binding sites of the matrix become saturated, and the target solute will flow directly through the matrix without net interaction. This is referred to as breakthrough. Thus, the emergence of the target solute from the matrix will result in a detected increase in ultra-violet absorption of the effluent. Thus, when solute concentration reaches a plateau indicating that the feed is simply flowing through the column without net solute interaction with the matrix surface, the solute concentration in the effluent equals the concentration in the feed.

When a non-diffusively bound chromatography matrix is used, or when liquid flow rates are slow relative to diffusion times, the above discussed phenomena result

in a graph with two well defined steps. That is, when an equilibrium concentration representative of the concentration of non-target solutes in the effluent is reached, a first well-defined plateau will result. This will be followed by a transition period indicated by a vertical line, or a line with a slope approaching the vertical, and a second plateau representative of the concentration of the target and non-target solutes together.

The difference between these equilibrium concentrations may be used to calculate the concentration of target solute in the sample as the difference between equilibrium concentration is directly proportional to the concentration of target solute in the sample. Furthermore, since the second plateau is indicative of the additive concentration of all solutes in the feed, that value can be obtained by monitoring the sample prior to the time it enters the matrix. Thus, all information necessary to calculate the target solute concentration is available as soon as a plateau in the breakthrough of non-target or contaminating solute is reached. The device is calibrated by passing through the solute detector known concentrations of pure target solute so that concentration units can be correlated directly with, e.g., absorbance units. The product of the difference between the sensed plateaus and the correlation factor equals the concentration of the target solute.

In an alternative embodiment, the method of the invention is used for detecting difference in the structural profile of a protein in separate samples. The phrase "structural profile", as used herein, refers to the particular mix of molecular species in a protein

solution which can vary from batch to batch or over time due to expression errors, differences in DNA sequence among the clones in a culture, truncation by proteases, or differences in post translational modification resulting in variations in conformation or derivatization. In this embodiment, the method comprises the steps of passing the samples through a matrix comprising immobilized binding sites which vary with respect to their binding properties to structural variants in the sample. For example, polyclonal antibodies may be used, cloned variants of which are specific for a particular epitope on a particular variant of the protein. Alternatively, a single type of binding site may be used which varies in binding affinity or specificity with variants of the protein to be analyzed. This procedure can produce a breakthrough function characteristic of the structural profile of the protein in the sample as the concentration of protein exiting the matrix is measured after at least some of the binding sites have been saturated with the protein. Comparing the characteristic functions of different samples permits indirect comparison of their structural makeup.

Since molecular subspecies in the protein mix have separate and distinct structural features, each subspecies has at least some unique epitopes. Each fraction of the binding protein in the matrix therefore will be capable of discriminating, (i.e., selectively binding) particular molecular subspecies, or of binding a molecular subspecies preferentially. Thus, as the protein sample is passed through the matrix, various of its subspecies reach equilibrium saturation, and thereafter break through into the effluent. If the protein concentration of effluent is monitored over

time, there is an interval over which protein concentration in the effluent increases from a baseline value, typically zero, to a value substantially identical to protein concentration in the feed. During the interval the protein concentration increases progressively in a way that is indicative of the particular structural profile of the protein sample. When this function is compared for separate protein samples, one can determine whether those samples have uniform structure. This method can be used, for example, to monitor a product stream periodically as a means of assuring that the product remains within a predetermined specification.

One may display the function of the sample as a plot of a parameter indicative of concentration (e.g., U.V. absorbance) against a parameter indicative of volume of sample exiting the matrix (e.g., time if flow rate is uniform). One may also elute the protein from the matrix, wash the matrix, and repeat and passing the measuring steps. The method is highly effective when the protein sample is an aqueous protein solution which has been at least partially purified. The method may involve passing the protein through the matrix by a means of a pressure gradient or a charge gradient.

The matrix preferably is a rigid, substantially non-microporous, particulate material having a hydrophilic surface, and preferably is a perfusive chromatography matrix. The matrix also may be defined by the interior surface of a capillary. Where the matrix comprises surface regions comprising immobilized protein A, protein G, and the binding protein is immunoglobulin, one can remove the binding sites from the matrix after each run, and reload the matrix with

fresh binding sites. Immunoglobulin and other types of protein binding sites also may be non-specifically adsorbed on a hydrophobic polymer matrix surface and removed with mixed organic/ionic stripping solutions.

It is necessary to the proper exploitation of the various embodiments of the present invention that a chromatographic or electrophoretic technique be used that results in a well-defined breakthrough. This can be achieved readily using essentially any matrix geometry provided the flow rate through the matrix is slow. At slow flow rates, the time required for solutes to diffuse into and out of the pores of the conventional HPLC or other chromatography medium is insufficient to destroy the development of a distinct concentration plateau in the effluent. However, at higher flow rates using conventional media, the concentration plateaus in the effluent typically are not discernible. This essentially means that, for desired high speed operation, non-diffusion bound chromatographic matrices should be used.

Also, the matrix should be as small as possible. The volume of sample that can be present in the matrix, coupled with the flow rate, dictate the time interval between introduction of the sample and breakthrough. Higher flow rates and small volume columns promote high speed analysis. This approach can resolute in assays being performed in periods of time substantially less than one minute and easily less than 10 seconds. For all practical purposes, these short time frames can be considered "real time" measurements.

The quantitative analysis technique is independent of flow rate, and does not require the target solute and the matrix to reach equilibrium. Thus, the sample

may be impelled through the matrix by any convenient method. The sample may be impelled through the matrix manually, e.g., using a syringe, by an electrically driven pump, or by a charge gradient as in electrophoresis.

It is a particular advantage of the invention that assays can be performed repeatedly without comprising the accuracy of the process. While an unknown subset of binding sites of the matrix may be degraded with repeated sequences of binding, elution, and reequilibration, the method of the invention generates information based on concentration differences of the target and non-target solutes. Thus, the availability of fewer binding sites will translate to earlier target solute breakthrough but will not give inaccurate indications of concentration.

The invention also affords a self checking capability. If detected concentration differs between the feed and the final effluent plateau, the system may be operating improperly. Self checking also can be implemented by washing the matrix after the final effluent plateau has been reached and then eluting the target solute. Integration of the detected pulse in the eluate will give an indication of the amount of bound target solute, which should correlate with the previous datum.

Another advantage of the invention is that it is very flexible. Consider, for example, a situation in which a sample having high concentration of target solute is passed through a matrix. This may result in almost immediate saturation of the binding sites of the matrix, and therefore, almost immediately breakthrough.

On a graph like that discussed above, the output will appear as a single vertical line followed by a horizontal plateau, giving no information about the concentration of target or non-target solute. To remedy this situation, the sample need only be diluted with buffer solution or the like. By diluting the sample breakthrough is delayed, thereby affording a clear distinction between the equilibrium concentration of the non-target solute in the effluent and the equilibrium concentration of the target and non-target solute together. If the amount of diluent is known, dilution does not adversely affect the precision or accuracy of the results. Assay of very dilute samples can also be conducted routinely. The only potentially negative effect on the system is that the time required to saturate the binding sites increases. This, of course, is a liability only for the self-checking aspect of the process, as the plateau reached after breakthrough of the target solute can be determined directly from the sample.

Another feature of the invention is that the binding sites on the matrix, e.g., monoclonal or polyclonal antibodies or other binding proteins, can be interchanged readily depending upon the identity of the target solute, using known techniques. This feature permits construction of a single matrix and assay device which can be customized for any target solute.

A further advantage of the invention is that it can be utilized on an extremely small scale. Even microliter sized samples can be analyzed. Moreover, rater than filling a traditional chromatography column with high surface area particles to serve as a matrix, one can coat binding protein on the inner surface of a

capillary tube. Passing a solution through the capillary tube can achieve the same results as those discussed above. It is contemplated that an assay device embodying the invention, including sample, eluant, and buffer ports, matrix channel ready to be activated with binding protein, detector for solute concentration in the effluent, and circuitry to convert the output of the detector to a meaningful form, all could be housed in a single module. Alternatively, an inexpensive matrix module can be produced for placement in a device comprising the other necessary components. The module can be discarded after a short useful life and then replaced. Alternatively, sets of modules each of which bind a different target solute will permit rapid adaptation of an assay apparatus for given target solute.

These and other advantages, objects, and features of the present invention will be more fully understood with reference to the following detailed description in conjunction with the attached drawing in which like reference characters indicate corresponding parts and in which:

Brief Description of the Drawing

FIG. 1 is a representative chromatogram generated in conjunction with diffusion bound chromatography at high throughput;

FIGS. 2 through 4 are various chromatograms illustrating the principles of the present invention;

FIG. 5 and 6 are schematic representations of two embodiments of apparatus embodying the invention, in

which like reference characters indicate corresponding parts;

FIGS. 7 and 8 are chromatograms generated using the analysis technique of the present invention for measuring the concentration of Immunoglobulin in solution using a Protein A column where BSA is a contaminant;

FIG. 9 is a chromatogram showing the results of an experiment involving the tertiary structural profile of mouse gamma globulin and demonstrating the feasibility of an embodiment of the invention; and

FIG. 10 is a representative calibration curve of milli absorbance units (mAu) versus protein (IgG) concentration in mg/ml.

Detailed Description of Preferred Embodiments

In its broadest aspects, the invention provides a method for monitoring the production of a solute based on subtractive frontal breakthrough analysis.

The concept is to exploit an affinity chromatography matrix to remove selectively at least one solute of interest from a solution, and to measure the equilibrium concentration of contaminating solutes in the effluent exiting the matrix. In a first embodiment, the next step involves determining the concentration of the target solute in the analyte sample from the difference between the sensed concentration of all solutes in the sample and the sensed concentration of the contaminants. In a second embodiment, the solute of interest is a protein,

particularly a purified recombinantly produced protein, comprising an unknown number of structural variants each of which vary at least subtly in their affinity for a particular binding protein, and have at least some unique epitopes. The protein sample, preferably substantially free of contaminants, is passed through a matrix comprising a single binding protein or immobilized polyclonal antibodies to the protein of interest. Variant protein molecules in the sample saturate the various clonal species of the polyclonal antibodies or compete for sites of attachment to a single type of binding site and then break through. Output monitoring produces a step-like plot of the breakthrough fronts characteristic of that particular sample.

Apparatus

Figures 5 and 6 schematically illustrate apparatus designed for implementing the process of the invention. Referring to Figure 5, a valve 10 directs through its output 12 either a sample from sample input 14, a buffer solution from reservoir 16 for washing and reequilibrating a chromatography matrix, or an eluent from reservoir 18 capable of inducing release of sorbed species from binding sites in a chromatography matrix. The output of valve 10 ultimately directs a selected solution through a chromatography matrix 20 of a nature hereinafter described in more detail, which comprises binding sites disposed about a surface and capable of selectively adsorbing an analyte or target solute sought to be determined. Optionally, interposed between valve 10 and matrix 20, as indicated in phantom at 22, is a solute concentration detector capable of providing a signal through line 24 representative of

the concentration of solutes in the sample. Detector 22 may be a conventional device of the type commonly used in chromatography equipment comprising, for example, a U.V. light source which provides a beam through a film of the sample and a U.V. detector which permits measurement of absorption by solutes in the sample. Liquid exiting matrix 20 enters detector 26 which also measures a parameter characteristic of solute concentration, this time in the effluent, and delivers a signal representative of that quantity through line 28. Lines 24 from detector 22 and line 28 from detector 26 enter electronic calculator means 30, where, for example, the difference between the sensed absorption maxima in detectors 22 and 26 is calculated, and that difference is used by multiplication with a conversion factor to determine target solute concentration. The concentration value may be delivered through line 32 to a display 34.

When detector 22 is omitted, the apparatus of Figure 5 operates slightly differently. Specifically, detector 26 detects a first plateau representative of the concentration of non-target solutes or contaminants exiting matrix 20, and at a later time, after breakthrough of the target solutes, detects total solute concentration. Data points representative of these sensed plateaus are delivered through line 28 to calculator means 30 and process as set forth above.

Figure 6 depicts another embodiment of the system of the invention. Its operation is conceptually identical to that a Figure 5 excepting that effluent from matrix 20 is returned to detector 22 via line 36. This permits a single detector to measure the total solute concentration in the sample prior to its

introduction into the matrix 20, and thereafter to measure the level of the plateau achieved in the effluent prior to breakthrough of the target solute. Design of this embodiment of the system may require inclusion of a liquid accumulator (not shown) in line 38 disposed between detector 22 and matrix 20, or some other means to insure that all sample has been removed from the detector 22 prior to the time solute concentration in the effluent reaches a plateau. Signals representatives of the solute concentrations sensed by detector 22 are transmitted through line 24 to calculator means 30 as disclosed above.

Calculator means 30 may be omitted if the purpose of the device is solely to monitor protein structure. In this case, the display 34 is adapted to display a plot of a function representative of protein concentration in the effluent versus a function representative of effluent volume. The display thus produces a curve characteristic of the structural profile of the protein sample which can serve as a "fingerprint" of the sample which will identify a given sample composition and change if the structural profile of the protein changes.

Assaying Solute Concentration

In the case of the each of the embodiments of Figures 5 and 6, prior to beginning an analysis, the system has been filled with a buffer 16 used to equilibrate matrix 20 and to assure no solute residues remain in detectors 22 or 26. To initiate an assay, the valve 10 is adjusted to permit sample 14 to be introduced into the system impelled by a pressure gradient created by a pump or syringe, or by means of a

charge gradient to promote electrophoretic movement through the matrix 20. In the optional embodiment of Figure 5 and in Figure 6, a data point indicative of the total solute concentration in the sample is sensed by detector 22. Thereafter, the sample enters the matrix 20. Target solute begins binding to the binding sites immobilized in the matrix; contaminants which do not bind pass through the matrix and emerge in the effluent. In the embodiment of Figure 5, the buildup of contaminants in the effluent is sensed by detector 26; in the embodiment of Figure 6, the buildup is sensed by return to detector 22. In both cases, prior to the time target solute saturates the binding sites in matrix 20 and begins breaking through into the effluent stream, the concentration of non-target solute(s) or contaminant(s) in the effluent stream reaches a plateau, and a signal indicative of the level of the plateau is passed to calculated 30.

At this point, all information needed to calculate the concentration of the target solute is available, and the assay is complete. However, as a check, flow through the system can be continued until the target solute breaks through matrix 20 and, together with the contaminants, produces a higher plateau which should be equal to the concentration sensed in the sample prior to its introduction into the matrix.

At this point, as an additional self check, if desired, valve 10 can be switched to direct buffer from reservoir 16 through the system, thereby washing detectors 22 and 26 and matrix 20 free of non-specifically adsorbed contaminants but leaving target solute non-covalently bonded to the binding sites in the matrix. After this wash step, valve 10 is again

switched to introduce eluent from reservoir 18 through the system. The eluent serves to elute the target solute from the matrix 20. The eluted target solute is detected by detector 22 (in the embodiment of Figure 6) or 26 (in the embodiment of Figure 5) as a pulse of solute. Integration of the pulse curve or other determination of the area under the curve gives an indication of the quantity of target solute bound during the assay which, again, can be correlated to the concentration derived previously.

From the foregoing, it will be appreciated that design and construction of all components of this system are well within the skill of the art. Indeed, many other configurations suitable for the practice of the process of the invention can be devised, and additional features incorporated as desired. For example, the system can be designed to have replaceable matrix modules, individual ones of which comprise binding sites specific for predetermined target solutes. Since accuracy of the assay is independent of flow rate, it matters not how one chooses to promote flow through the system. Thus, for example, a pump may be placed anywhere in the fluid flow line. Alternatively, the sample may be placed in a syringe and simply rammed through the system.

The calculator means or processor 30 can take various forms, and indeed, in the broader aspects of the invention, is not required. A conventional plotter attached to detector 22 and/or 26 would permit an operator of a production or purification s··-tem to determine visually by observing plural consecutive plots whether concentration of the target solute and/or the impurities is changing with time or is constant.

However, calculator 30 may include means for storing signals representative of data points indicative of the sensed solute concentration ratios, and correlation factors, and an arithmetic calculation module which calculates target solute concentration and/or contaminant solute concentration. These data may be displayed digitally in display 34 after each assay. Alternatively, the data may be used to produce a plot of target solute concentration over time, or other desired indication of the state of the system, as a record of the dynamic behavior of the system under analysis.

A chromatogram is generated by measuring and charting a characteristic of the effluent that varies in proportion to the concentration of detectable solute in the effluent. In a typical application, commonly used in commercial chromatography equipment, ultra-violet radiation is passed through the effluent and the degree of ultra-violet absorption is charted. Absorption of U.V. light in such systems is proportional to solute concentration, provided the solute is absorptive of this wavelength. It should be understood, however, that any characteristic of the effluent which is representative of the concentrations of analyte and impurities therein can be monitored for purposes of the present invention.

The abcissa of the chromatogram of Figure 1 indicates time, and the ordinate absorption. For illustrative purposes the graph is divided into five periods which are labelled A, B, C, D, and E. The periods define stages of solute concentration in the effluent during a chromatographic loading cycle that might be encountered when passing a sample through a

conventional affinity chromatography matrix housed in a column at a high rate, e.g., 1800 cm/hr. It is easy to see that due to poor resolution the boundaries between periods must be drawn rather arbitrarily.

Initial period A represents the condition where the effluent consists entirely of buffer. When the effluent begins to include impurities from the sample, the solute concentration begins to rise as shown in period B. Eventually, an equilibrium concentration will be reached as depicted in period C. This will occur when non-specific binding (if any) of impurities to the matrix has stopped and target solute is being retained by binding to the matrix so that the concentration of impurities in the feed is equal to the concentrations of impurities in the effluent. As sample continues to flow through the matrix, the target solute begins to saturate the binding sites of the matrix. This results in the emergence of target solute in a gradually increasing concentration in the effluent, commonly referred to as "breakthrough", illustrated in period D. When the binding sites are completely saturated (period E), the sample merely flows through the matrix and the concentration of solute in the effluent is equal to the concentration of solute in the feed.

Note that the "plateau" of period C is the critical information necessary to calculate the concentration of the target solute, but that the height of the plateau, and its boundaries, are far from distinct. For samples containing multiple solute of differing physical properties, the chromatogram can be far less informative, and the faster one passes the sample through the matrix, generally the more the critical plateau is marked by band spreading.

There is shown in FIG. 2 a chromatogram typical of that obtained by passing the sample very slowly through the matrix. The single most significant distinction between the graphs of FIGS. 1 and 2 is that the latter has sharply defined breakthrough points and equilibrium levels. Period A' of FIG. 2 corresponds to period A of FIG. 1 and is representative of the period over which buffer alone constitutes the effluent. FIG. 2 shows that the matrix becomes saturated with impurities due to non-specific binding over a short interval so that an equilibrium concentration of impurities is reached in period C' at a very well defined point in time. This is represented in the figure as breakthrough point B'. The equilibrium concentration of period C' will be maintained as analyte contained in the solution is loaded onto the binding sites of the matrix until those binding sites become saturated. When this occurs, a second breakthrough point D' will be reached wherein the concentration of the analyte in the effluent will become equal to the concentration of the analyte in the feed solution. The concentration of the analyte and impurities together will be directly proportional to the equilibrium concentration of period E' which follows the second breakthrough step D'. The difference, therefore, between the height of plateau E' and the height of plateau C' can be used to calculate the concentration of analyte in the feed solution.

Additionally, if the capacity of the matrix is known, by monitoring the amount of solution passed through the matrix before the breakthrough step D' occurs, the concentration of analyte in the solution also can be determined. Since, however, over repeated uses the binding capacity (the number of binding sites

in the matrix) will decrease, it will more often be the case that the concentration of analyte in the solution will be determined based upon the principles discussed above. The concentration so determined, therefore, can be used in conjunction with the timing of the breakthrough step D' to determine how many binding sites remain in the matrix.

Line F' in FIG. 2 represents the point at which solution has ceased being passed through the matrix, and the effluent once again comprises only buffer. A third way to determine the amount of analyte in the solution is during desorption of the analyte from the matrix by way of passing an eluent through the matrix to free the analyte from the binding sites. This process is represented in the figure by the behavior of the chromatogram during period G'. The area under the curve in this period is directly proportional to the amount of analyte bound to the matrix as of the breakthrough point D'. It is clearly possible, therefore, to check the accuracy of the determination of target solute concentration made based on the height of step D' to that determination made based upon the area under the curve during period G'.

Figure 3 shows a chromatogram of the type which can be produced in the apparatus of Figure 5 with optional detector 22, or in the apparatus of Figure 6. Period A' represents the interval when detector 22 is measuring total solute content in the sample prior to its entry into matrix 20. After leaving detector 22 the sample enters matrix 20, and during the time the sample is displacing buffer in the matrix, the concentration of effluent from the matrix shows a solute free state as illustrated during interval B'.

Impurities break through at C' and their concentration is represented at interval D' in Figure 3. As flow through the matrix proceeds, target solute breaks through at E', and total solute concentration in the eluent, indicated at F', equals the concentration indicated by the interval A'. The data necessary to know target solute concentration is in hand as soon as the level of plateau D' is known with confidence, and is here indicated by way of example by a vertical dotted line. The time it takes for the level D' to be established is dependent on flow rate and on the volume of the column, which is proportional to length B'. Small volume columns which can be run at high flow rates are therefore preferred for rapid analysis.

With repeated use the chromatography matrix will break down in the sense that its capacity will decrease. This will not, however, affect the accuracy of the data generated in accordance with the principles of the present invention. All that occurs is that the length of plateau C' in Figure 2 or D' in Figure 3 becomes shorter, as target solute breaks through sooner. Neither the height of the breakthrough plateau representative of the concentration of the impurities nor the solute concentration maxima change, and accuracy is not compromised.

It is possible, however, for a problem to develop if, in relation to the concentration of the analyte, there is an inadequate number of binding sites. That is, if the concentration of analyte in the solution is so high with respect to the number of available binding sites, or fluid velocity so high, that the binding sites become saturated almost immediately, a condition will result wherein, rather than displaying two well

defined steps, a chromatogram will show only one step. This situation is portrayed in the chromatogram depicted in FIG. 4, wherein a single breakthrough point X represents the simultaneous saturation of both analyte and impurities in the chromatography matrix. In this situation it is obviously impossible to discern equilibrium concentration levels. While the area under the curve in period G' will still be proportional to the amount of analyte captured by the matrix at the point of saturation, saturation has occurred so quickly that reliable determination of concentration based on saturation cannot be made. To remedy this problem, the feed solution need only be diluted with, e.g., buffer, prior to being passed through the matrix, so that breakthrough of the non-target solute can be distinguished. After dilution, a chromatogram such as that depicted in either FIG. 2 or 3 will be generated wherein an equilibrium concentration of impurities is established in the effluent before any analyte appears in the effluent.

As has been mentioned throughout this description, a preferred aspect of the present invention involves high speed assays, e.g., less than 10 seconds. The above discussed analyses can be performed in periods substantially shorter than one minute, often shorter than 30 seconds, and frequently less than 10 seconds, if one employs a small volume column containing a matrix medium of the type described below.

The present invention also helps to increase the speed with which assays are carried out by being able to provide meaningful data with only a very small sample of feed solution. For example, assays routinely can be performed on microliter sized samples. For this

purpose, the chromatography matrix can be supported in a column having suitable dimensions. For extremely small samples, it is possible to pass the sample through a capillary tube having an inner surface coating of binding protein.

One currently preferred matrix is non-porous (or very low porosity) affinity-based silica particles. A particularly advantageous matrix medium is POROS™ brand column packing materials which may be obtained commercially from PerSeptive BioSystems, Inc. (Cambridge, MA). These materials are produced through suspension polymerization techniques and classified to the desired particle size range. POROS™ columns have been shown to have significantly reduced band spreading in high speed assays, thereby allowing analysis according to the practice of the present invention to be performed in extremely short periods of time.

The invention will be understood further from the following nonlimiting examples.

EXAMPLE 1.

The subtractive frontal analysis technique is demonstrated using a Protein A column to measure the concentration of human Immunoglobulin in solution. Various concentrations and purity levels of IgG were analyzed using an HP 1090 liquid chromatograph (available from Hewlett Packard, Waldbronn, GmbH) and a "chem station" (available from Hewlett Packard). Pure samples of human IgG (available from Sigma Chemical Company, St. Louis, Missouri) are prepared in concentrations ranging from 0.01 - 10 mg/ml. These samples are first pumped into a detector flow cell

without a column in line, and the absorbance at equilibrium is quantified to obtain a calibration curve. Next 250 µl injections of IgG samples contaminated with various amounts of BSA (Sigma Chemical Company) are mixed in 10 mM sodium phosphate and 150 mM sodium chloride to pH 7.4 and run on a 2.1 x 30 mm POROS™ A/M column, a commercially available perfusive column comprising 20 µm polystyrene/divinyl benzene beads with a pellicular coating of protein A. As an example, absorbance plateaus of the output of 0.5 mg/ml IgG mixed with 0.5 mg/ml BSA at flow rates of 1 ml/min and 0.1 ml/min are shown in Figures 7 and 8, respectively. The profiles indicate breakthrough of BSA and IgG are clearly distinguishable.

The accuracy of this system may be checked by analyzing samples of known concentration. A calibration curve relating the detector absorbance and concentration for a single component is needed to obtain meaningful comparisons with the known concentration in a sample mixture. Figure 10 shows such a calibration plot for human gamma globulin using the 1090 diode array detector at 280 nm. Two independent sets of experiments yielded the data and correlation shown. Human gamma globulin is about 92.4% pure, as determined by subtractive frontal analysis. Therefore this factor has to be included when converting a measured absorbance to equivalent IgG concentration. The calibration plot also reveals the linear range to be up to about 4 mg/ml for pure IgG or about 3000 mAu total for a mixture.

A set of experiments were conducted using mixtures of different concentration and purity of IgG (using BSA as contaminant). Comparing the expected absorbance against the actual column absorbance and correcting for

the non-retained plateau value as shown in Table 1 and described below allows an accurate estimation of the IgG concentration.

As is evident from the data, the prototype device can detect varying IgG concentration in the presence of varying amounts of contaminating protein, here BSA. Certain experiments detailed in Table 2 revealed conditions under which the method is less accurate. Errors result from a number of factors, including the calibration plot, the value of the absorbance at the plateau, and measurements outside the linear range. In addition, in these experiments there may also be errors in the measurement of amounts of proteins used in the test mixture. Errors due to the calibration are found in the examples in Table 1. Errors in estimating the absorbance of the front emerging from the column (i.e., the non-bound contaminant) are magnified for cases where the IgG purity is 10% or less as shown in Table 2. For example a 5% error in the estimate of the plateau absorbance yields a 45% error in the IgG concentration estimate for a sample of 10% pure IgG. The same 5% error in the plateau only leads to a 5% error in the concentration estimate for a sample of 50% purity. Errors caused by exceeding the linear range are evident in Table 2. In this case, a simple solution would be to dilute the sample appropriately. Alternatively, the use of a slightly different wavelength (higher or lower) than 280 nm will produce a weaker signal response and can be used for samples of high concentration.

These data, taken collectively, demonstrate the utility and feasibility of the assay procedure. Routine engineering principles may be used in the design of a commercial device to improve accuracy and

ease of use. For example, computing means 30 may be programmed to require 5 or 10 consecutive readings over an appropriate time interval to be within some small margin of error before a "plateau" is recognized and recorded.

Table 1

A IgG mg/ml	B BSA mg/ml	C no col mAU	D column mAU	E exp IgG	F conc IgG	G % VAR IgG
0.5	0.5	530	200	570	0.49	1.61
0.5	0.055	350	41	386	0.46	7.87
1	1	1110	395	1139	1.07	6.59
1	0.111	725	84	772	0.96	4.45
1.5	1.5	1595	605	1709	1.48	1.61
1.5	0.156	1170	135	1153	1.54	2.86
0.05	0.05	53	18	57	0.05	4.35
0.05	0.0056	37	3	39	0.05	1.37
0.1	0.1	106	37	114	0.10	2.86
0.1	0.011	78	8	77	0.10	4.35
0.2	0.2	221	79	228	0.21	5.84
0.2	0.022	144	18	154	0.19	6.09
0.4	0.4	425	158	456	0.40	0.50
0.4	0.044	294	37	309	0.38	4.22
0.1	0.2333	163	95	169	0.10	1.37
0.2	0.4666	322	178	338	0.21	7.33
0.4	0.9333	645	348	676	0.44	10.68
0.5	2	1091	745	1189	0.52	3.16
0.3	2.7	1170	964	1333	0.31	2.36
0.15	1.35	610	496	666	0.17	13.29
0.075	0.675	273	226	333	0.07	6.58
				avg deviation		4.73

Table 2

0.05	0.45	200	160	222	0.06	19.3
0.1	1.9	800	718	857	0.12	22.2
1	9	2970	2680	4443	0.43	56.8
1.5	13.5	3020	2900	6665	0.18	88.1

Table Legend

A: Known IgG concentration (C_{IgG}) in mg/ml;
B: Known BSA concentration (C_{BSA}) in mg/ml;
C: Absorbance in milli absorbance units (mAU) without the column in line (corresponding to effluent after saturation of all binding sites in column, or height of second plateau);
D: Absorbance in mAU with the column in line (corresponding to absorbance of BSA alone - first plateau caused by BSA breakthrough);
E: "Expected" value of absorbance without the column in line calculated by:
$(C_{IgG})(726\ mAU) + (C_{BSA})(413\ mAU)$ where 726 mAU is IgG absorbance factor from calibrator curve of Fig. 10 and 413 is BSA absorbance from calibration curve (slope);
F: Detected IgG concentration - (C-D/726 X 0.924) where 0.924 is the percent IgG absorbed); and
G: $\frac{\text{Detected IgG - Actual IgG}}{\text{Actual IgG}} \times 100.$

While there is some band spreading in the second IgG-containing front of Figure 7, the 10X increase in speed between the runs represented by Figs. 7 and 8 significantly offsets the disadvantage of the delay of the second plateau. In fact, as discussed above, the second plateau is not necessary for calculating the concentration of the sample where the original absorbance of the test solution is known, making the band spreading of the second front irrelevant.

EXAMPLE 2

To demonstrate the practice of the invention in characterizing the structural profile of a protein, a Protein A column of the type described above is loaded with Mouse Gamma Globulin (Sigma). Mouse gamma globulin actually contains several subclasses of IgG which vary with respect to their binding affinity for Protein A. If a sample of 2 mg/ml protein in 10 ml PBS plus 1% MeOH is run on a 2.1 x 30 mm column at a flow rate of 0.2 m./mn followed by elution with 0.15 M NaCL + 2% Acetic Acid + 1% MeOH, a frontal chromatogram as shown in Figure 9 is produced. The shape of the curve is indicative of the affinity of the various IgG species in the sample for protein A on the POROS A/M column, and changes in the structural profile of protein in the sample will induce variations in this curve. Thus, the curve constitutes a "fingerprint" uniquely identifying this particular mix and condition of IgG species, and repetition of the procedure with other samples will produce a curve permitting one to compare the structural profile of the samples.

Other embodiments are within the following claims.

What is claimed is:

1. Method for assay of a sample comprising one or more impurities and a target solute, the method comprising the steps of:

A) passing the sample through a matrix having binding sites specific for the target solute to produce, while target solute is loaded onto the matrix, an effluent, substantially free of said target solute, which reaches a first equilibrium concentration representative of the concentration of the impurities;

B) obtaining a first data point representative of said first equilibrium concentration and a second data point representative of the additive concentration of solutes in said sample; and

C) determining the difference between said first and second data points, the difference being proportional to the concentration of the analyte in the sample.

2. The method of claim 1 wherein said second data point is obtained by monitoring the solute concentration in said sample before it is passed through said matrix.

3. The method of claim 1 wherein said second data point is obtained from the effluent after the matrix becomes saturated with a target solute.

4. The method of claim 1 wherein the step of obtaining said first and second data points includes the step of:

monitoring the ultra-violet absorption of the effluent, the value of said absorption being proportional to the concentration of impurities in the sample.

5. The method of claim 1 wherein the solutes are proteins.

6. The method of claim 1 wherein said binding sites are antibody binding sites.

7. The method of claim 1 wherein said matrix comprises Protein A, Protein G, an analog or fragment thereof, and said binding sites comprise immunoglobulins.

8. The method of claim 1 wherein the matrix comprises rigid, substantially non-porous particulate material.

9. The method of claim 1 wherein the matrix is a perfusive chromatography matrix.

10. The method of claim 1 wherein the step of determining the difference between said first and second data points includes the steps of:

generating a first electronic signal representative of the value of said first data point;

generating a second electronic signal representative of the value of said second data point;

electronically comparing said first and second signals; and

electronically calculating the concentration of said target solute based on said electronic comparison.

11. The method of claim 10 comprising the additional step of displaying the concentration of said target solute.

12. The method of claim 1 comprising the additional steps of:

washing the matrix;

eluting target solute bound to the matrix; and

detecting the quantity of target solute in the eluate.

13. The method of claim 1 comprising the additional steps of:

eluting the target solute from the matrix;

washing the matrix; and

repeating steps A, B and C with a second sample.

14. The method of claim 1 wherein the matrix comprises reversibly bound binding sites, and the method comprises the additional steps of:

removing the binding sites from said matrix; and

reloading the matrix with fresh binding sites.

15. The method of claim 1 comprising the additional step of diluting the sample prior to step A to produce a solute concentration suitable to permit an

equilibrium concentration of said impurities to be established in the effluent before appearance of target solute in the effluent.

16. Apparatus for assay of a target solute in a sample comprising plural solutes including one or more impurities, the apparatus comprising:

a matrix having binding sites specific for the target solute;

means for passing the sample through the matrix to produce an effluent which reaches a first equilibrium concentration, while target solute is loaded onto the matrix, representative of the concentration of the impurities;

means for obtaining a first data point representative of the first equilibrium concentration;

means for determining the difference between said first data point and a second data point representative of the additive concentration of solutes in the sample, said difference being proportional to the concentration of the target solute in the sample.

17. The apparatus of claim 16 wherein said means for passing includes means for generating a pressure gradient across the matrix.

18. The apparatus of claim 16 wherein said means for passing comprises means for generating a charge gradient across the matrix.

19. The apparatus of claim 16, wherein said matrix comprises rigid, substantially nonporous particulate material.

20. The apparatus of claim 16 wherein said matrix is a perfusive chromatography matrix.

21. The apparatus of claim 16, wherein said matrix comprises Protein A, Protein G, analogs or fragments thereof.

22. The apparatus of claim 16 wherein said means for obtaining includes means for monitoring the ultra-violet absorption of said effluent.

23. The apparatus of claim 16 wherein said matrix is contained within a column.

24. The apparatus of claim 16 wherein said matrix is disposed on an inner surface of a capillary tube.

25. The apparatus of claim 16 wherein said means for determining the difference between said first and second data points includes a calculator means including;

means for generating a first electronic signal representative of the value of said first data point;

means for generating a second electronic signal representative of the value of said second data point;

means for determining the difference between said first and second data points; and

means for calculating target solute concentration from said difference.

26. The apparatus of claim 25 further comprising means for displaying said concentration.

27. The apparatus of claim 16 further comprising means for passing an eluant through said matrix.

28. The apparatus of claim 16 further comprising means for equilibrating said matrix after step C whereby steps A, B and C may be repeated with a second sample.

29. A method characterizing the structural profile of a protein sample comprising a mixture of structural variants, the method comprising:

passing the sample through a matrix comprising immobilized binding sites for epitopes on said protein, and, after saturation of at least some of said binding sites,

measuring a parameter indicative of the concentration of protein exiting the matrix for a time sufficient to obtain a breakthrough function characteristic of the structural profile of the protein in the sample.

30. A method of detecting differences in the structural profile of protein in separate samples, each of which comprise a mixture of structural variants, the method comprising:

passing the samples through a matrix comprising immobilized binding sites for epitopes on said protein,

measuring a parameter indicative of the concentration of protein exiting the matrix during an interval after at least some of the binding sites have been saturated with said protein to obtain a

breakthrough function characteristic of the structural profile of the protein in the sample, and

comparing the characteristic breakthrough functions of the samples.

31. The method of claim 29 and 30 comprising the additional step of displaying the function as a plot of said parameter indicative of concentration versus a parameter indicative of volume of sample exiting the matrix.

32. The method of claim 29 or 30 wherein the protein sample is a purified aqueous protein solution.

33. The method of claim 29 or 30 wherein the binding sites comprise polyclonal antibodies raised against a said protein sample.

34. The method of claim 29 or 30 wherein the binding sites comprise a single type of binding site having differing binding properties for protein variants in said protein sample.

35. The method of claim 29 or 30 wherein the matrix comprises rigid, substantially non-microporous, particulate material having a hydrophilic surface.

36. The method of claim 29 or 30 wherein the matrix is a perfusive chromatography matrix.

37. The method of claim 29 or 30 wherein the matrix comprises the interior surface of a capillary tube.

38. The method of claim 29 comprising the additional steps of:

eluting the protein from the matrix;
washing the matrix; and
repeating said passing and measuring steps.

39. The method of claim 38 wherein the matrix comprises reversibly bound binding sites, said method comprising the additional steps of:

removing the binding sites from the matrix; and
reloading the matrix with fresh binding sites.

40. The method of claim 29 or 30 wherein said passing step is effected by a pressure gradient or a charge gradient.

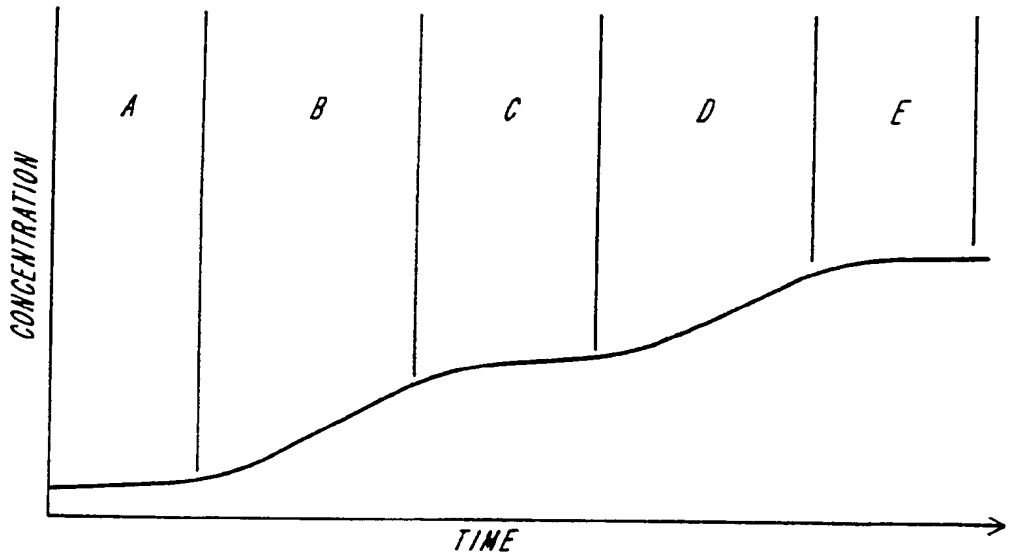

FIG. 1

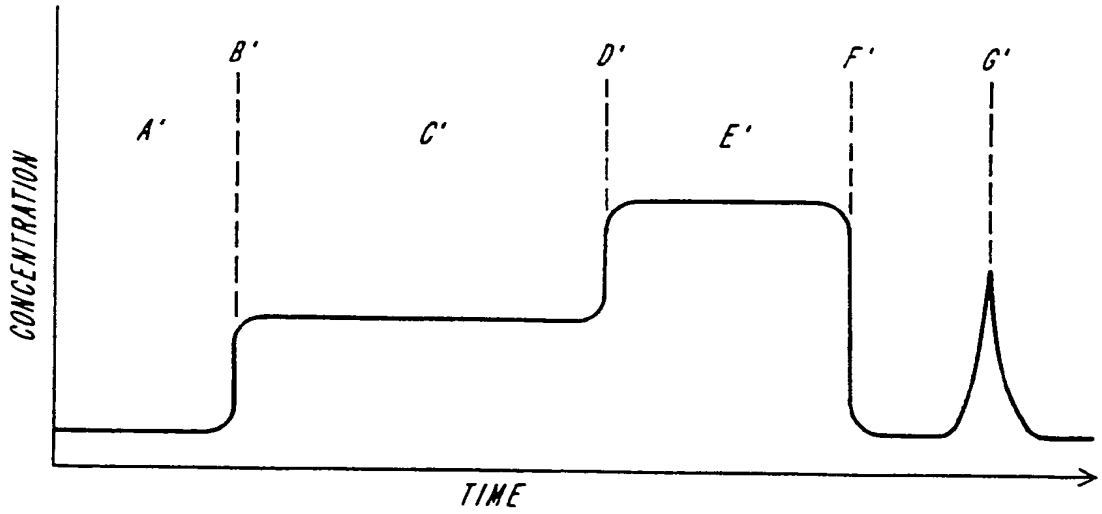

FIG. 2

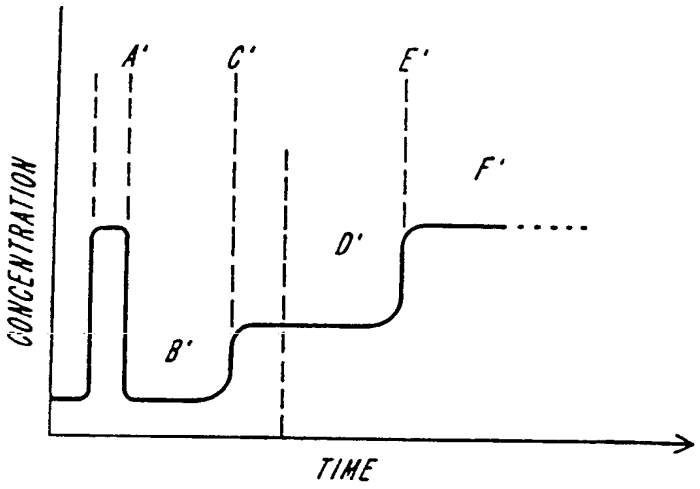

FIG. 3

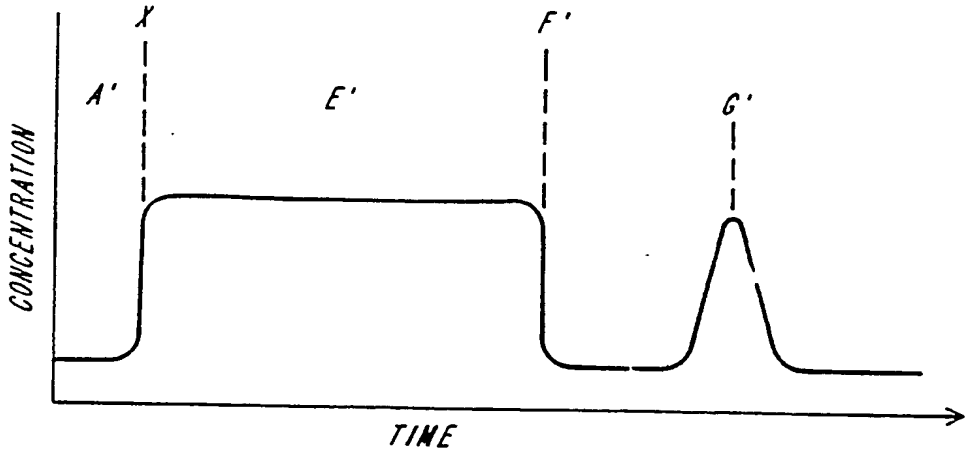

FIG. 4

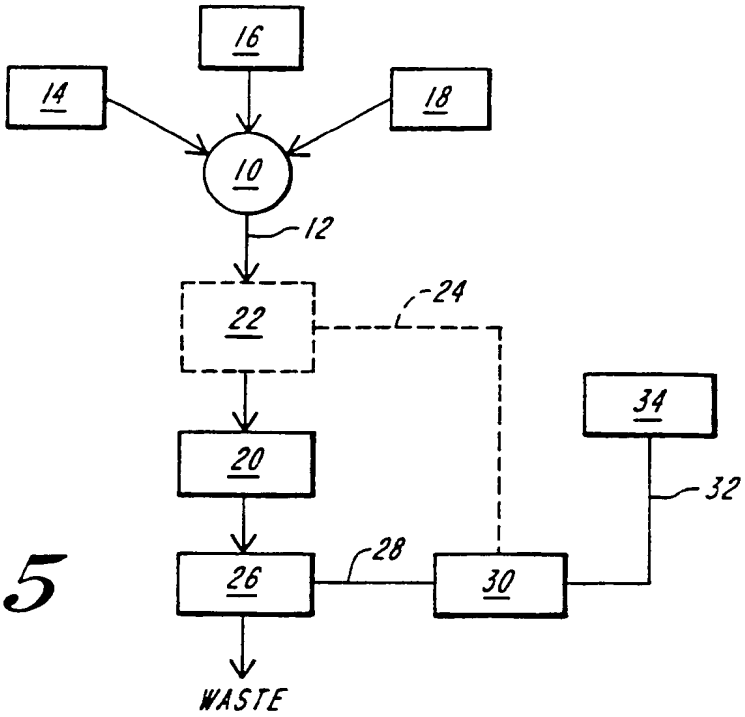

FIG. 5

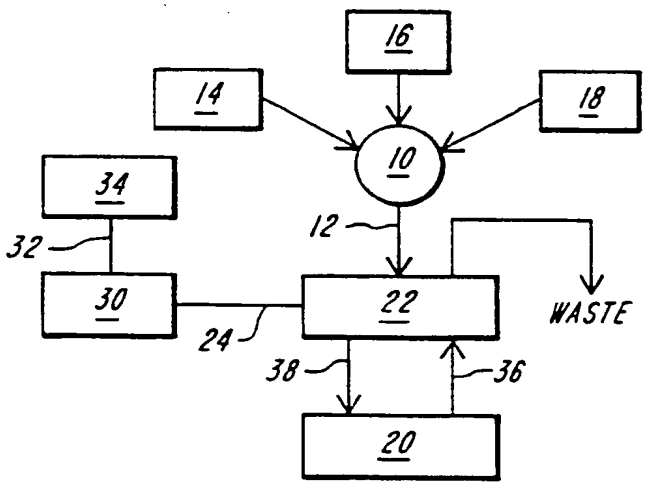

FIG. 6

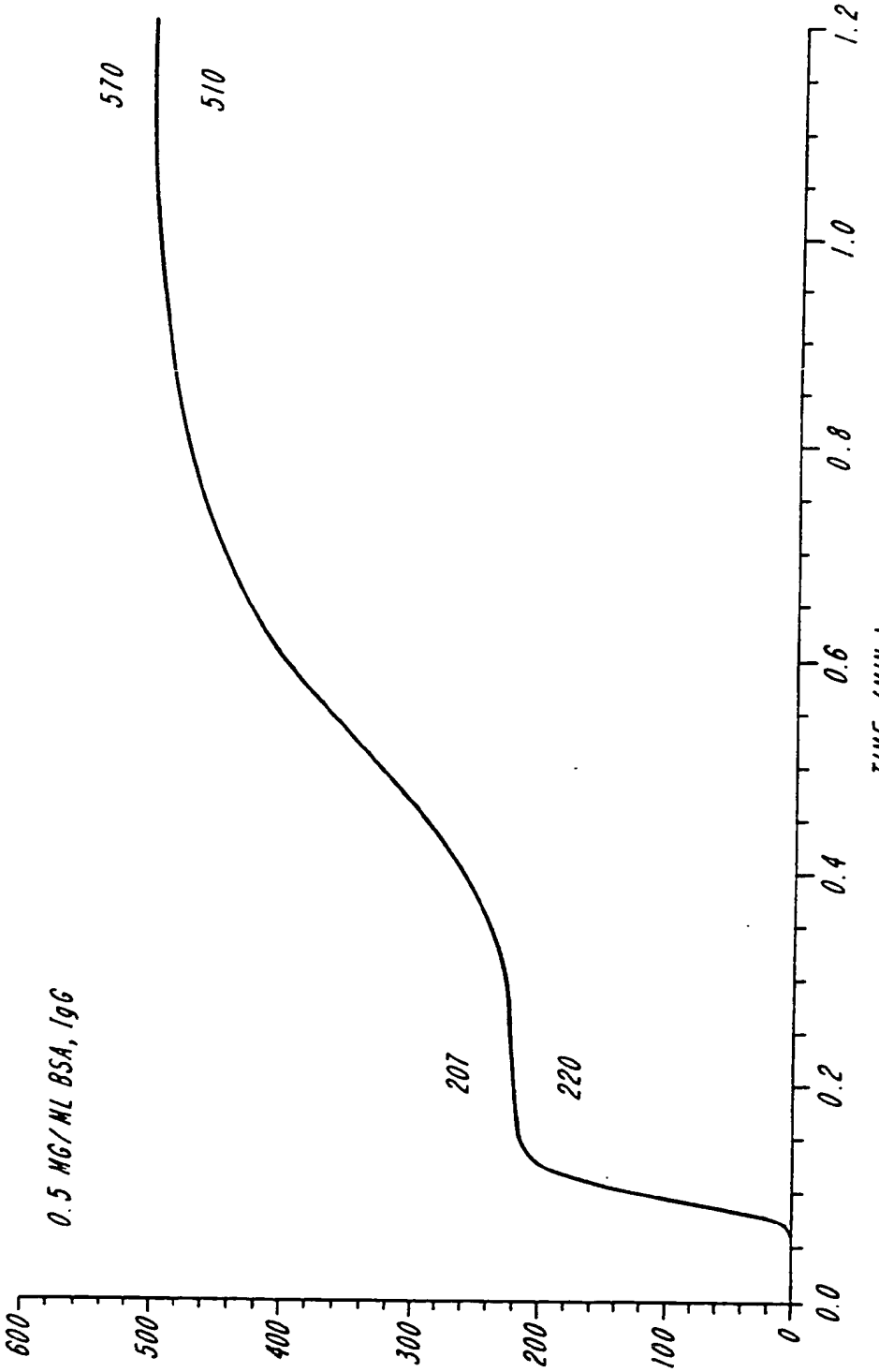

FIG. 7

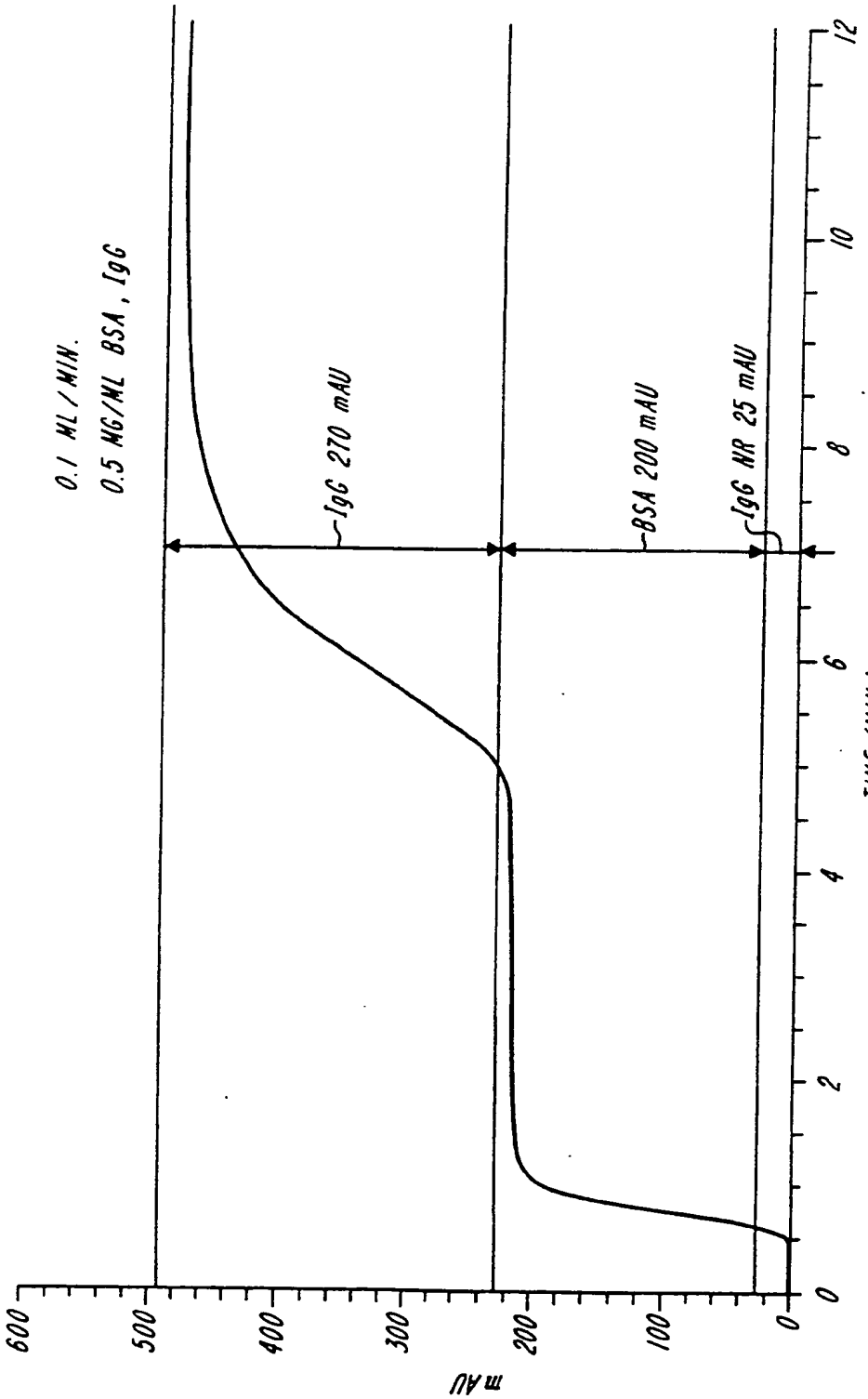

FIG. 8

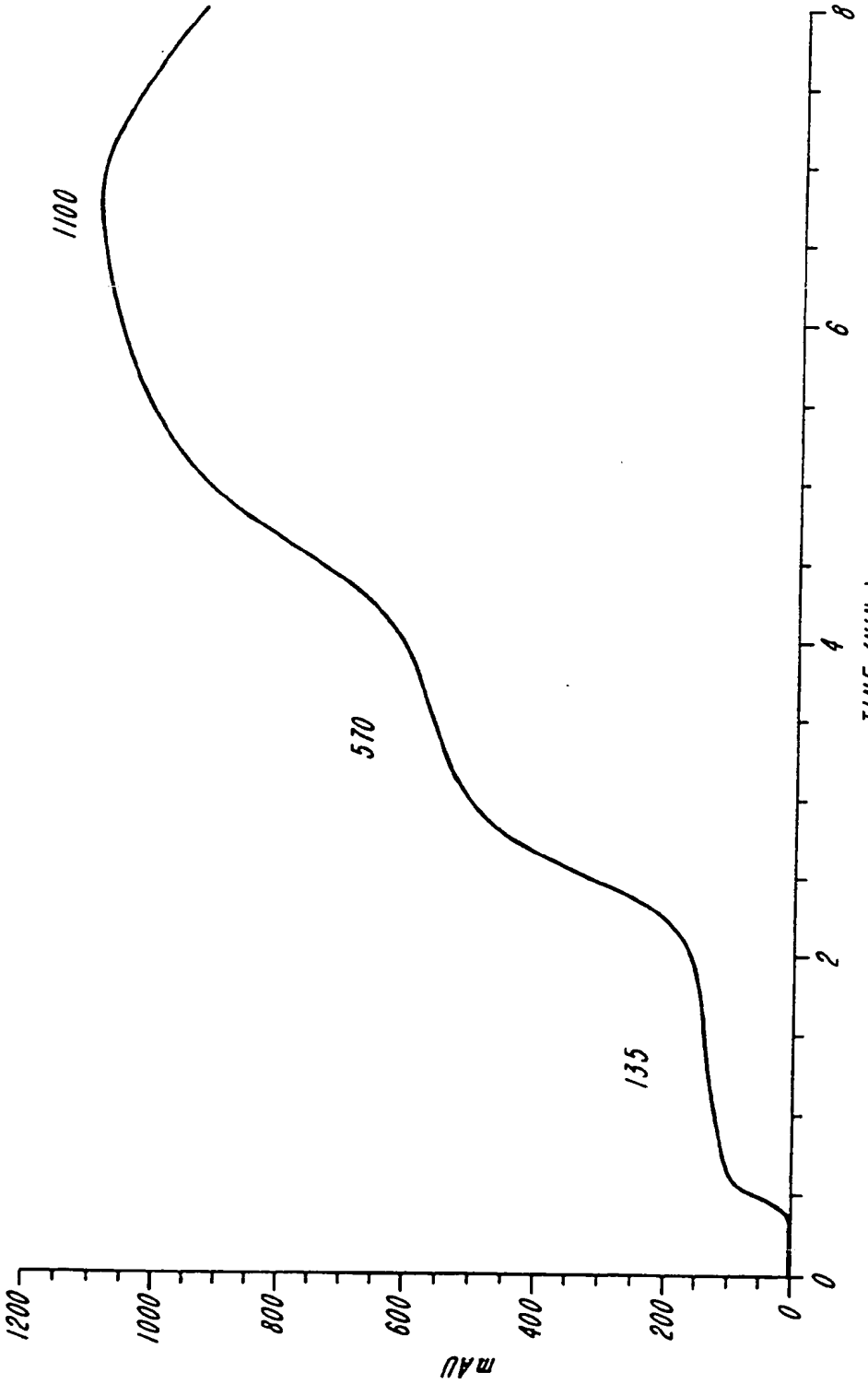

FIG. 9

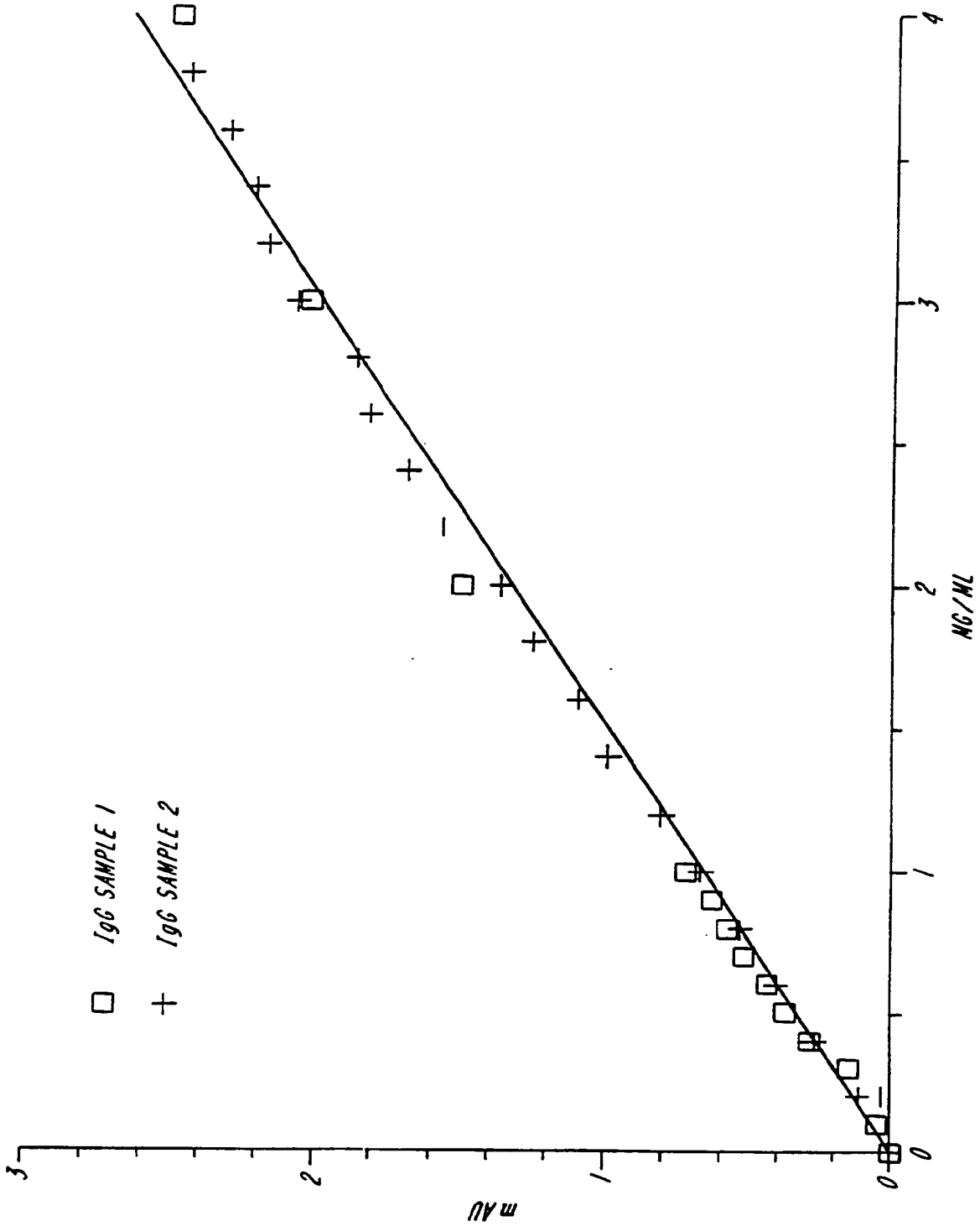

FIG. 10

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(54) Title: QUANTITATIVE ANALYSIS AND MONITORING OF PROTEIN STRUCTURE BY SUBTRACTIVE CHROMATOGRAPHY

(57) Abstract

Methods are provided for making real-time determinations of the concentration and for monitoring the structural profile of a target solute in a solution. Non-diffusion bound affinity chromatographic techniques are used to generate chromatograms having sharply defined breakthrough curves. Based on the difference between the sensed equilibrium concentration of the impurities which break through the matrix and the concentration of all solutes in the sample, the concentration of the target solute in a solution can be determined. Alternatively, a chromatogram representative of the structural profile of a protein in a sample comprising a mixture of structural variants may be produced using the method of the invention. The assays may be carried out in real-time so that production or purification of a valuable substance such as a pharmaceutical recombinant protein or the like can be monitored meaningfully.

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+ Any designation f "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in ther States of the former Sovi t Union.

INTERNATIONAL SEARCH REPORT

PCT/US 91/05544

International Application

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 G01N30/88; G01N30/62

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	G01N ; B01D

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
A	BIOTECHNOLOGY vol. 8, no. 3, March 1990, NEW YORK US pages 203 - 206; AFEYAN ET AL.: 'perfusion chromatography an approach to purifying biomolecules' see page 204, column 3 - page 205, column 1, paragraph 2 ---	1,29,30
A	WO,A,9 006 516 (PRIMUS CORP.) 14 June 1990 see page 1, line 5 - line 11 see page 5, line 22 - page 7, line 11 see page 8, line 21 - page 9, line 21 --- -/—	1,16,29, 30

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
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III. D CUMENTS C NSIDERED T BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	JOURNAL OF CHROMATOGRAPHY. vol. 512, 20 July 1990, AMSTERDAM NL pages 365 - 376; LLOYD ET AL.: 'preparative high-performance liquid chromatography on a unique high-speed macroporous resign' see page 373 see page 375, paragraph 4	29,30
A	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 259, no. 6, 1984, BALTIMORE US pages 3796 - 3799; YIN CHANG LIU ET AL.: 'quantitative analysis of protein: immobilized dye interaction' see page 3796, column 2, paragraph 3 -paragraph 5	1,29,30
A	US,A,4 204 424 (WALKER) 27 May 1980 see column 3, line 9 - line 42; figure 1	1,16
A	EP,A,0 141 259 (EVIROMENTAL SCIENCES ASSOC.) 15 May 1985 see page 6, line 15 - line 45; figures 1,4 see page 9, line 5 - line 8 see page 9, line 11 - line 19 see page 11, line 3 - line 12	1,16

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US SA 9105544 51212

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 03/03/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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